nature portfolio

Peer Review File

Cross-reactive human antibody responses to H5N1 influenza virus neuraminidase are shaped by immune history

Corresponding Author: Dr Scott Hensley

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this study, the authors measured NA inhibition antibodies against multiple H5N1 viruses using sera from individuals born in different time periods and found that individuals primed in childhood with H1N1 viruses were more likely to possess higher levels of antibodies that cross-react with the NA of H5N1 viruses compared to individuals primed in childhood with H2N2 or H3N2 viruses. While young children rarely possessed cross-reactive NA antibodies, childhood infections with contemporary H1N1, but not H3N2, viruses can elicit them, suggesting that immune history greatly impacts the generation of cross-reactive NA antibodies that can inhibit H5N1 viruses.

This study provides interesting insights on the potential explanations of age-related N1 cross-reactivity.

Comments for consideration:
1. The authors are concluding the effect of imprinting from a wide range of age cohorts and birth years, but the sample sizes used are rather small and the manuscript lacks details on the basic information of the sera used (e.g. birth years of the cohorts, sample sizes in each of the birth cohorts, and how the sera were selected for analysis), thus make it difficult to assess the representativeness of the results. It appeared that the authors used 3 groups of sera:
1). 155 sera collected in 2017 with birth year 1927-2016, no details of the breakdown in each category of the age cohorts.
2). Serum samples collected in 2005, numbers unknown with no details
3). Seasonal influenza infection sera.

Suggest the authors include a table to describe the critical parameters of the sera used, including the sample sizes in each of the birth cohorts studied and acknowledge the small sample size as a study limitation.

2. The authors can reference a recent study reported by ZN Li et al (Population Immunity to Hemagglutinin Head, Stalk and Neuraminidase of Highly Pathogenic Avian Influenza 2.3.4.4b A(H5N1) viruses in the United States and the Impact of Seasonal Influenza on A(H5N1) Immunity | medRxiv). This study analyzed a larger sample size and investigated broader age groups and reported the prevalence of NA antibodies of A(H1N1)pdm09 and 2.3.4.4b H5N1 viruses with similar age-related patterns. A(H1N1)pdm09 virus infection in adults elicited significant rise of cross-reactive NAI antibody response to H5N1. In addition, they observed a significant rise of cross-reactive NAI antibody response to the NA of H5N1 in different age cohorts following recent vaccinations. This study also explored the similarity between the N1 NA epitopes of H5N1 and H1N1 virus which can contribute to the N1-imprinting effect.

3. For the same set of serum samples used for Figure 1, suggest the authors test N2 NAI antibodies as a specificity analysis for the source of NA imprinting. It would also be interesting to see if the prevalence of N2 antibodies is correlated with N2-imprinting, and whether the two NA imprinting pathways can interact and affect each other. This may provide insights into the peak titers for people born during the 1990s (Figure 1) which as the authors discussed, cannot be explained by imprinting probabilities alone.

4. In line 94-110, should describe how “N1 imprinting probabilities” is defined and how it differs from the “birth year” in the analysis, assuming that the N1 imprinting probabilities are also determined based upon the birth years.

Reviewer #2

(Remarks to the Author)
In this Brief Communication, Ort et al. demonstrate that individuals primed in childhood with H1N1 influenza viruses exhibit higher levels of antibodies cross-reactive with clade 2.3.4.4b H5N1 neuraminidase than those primed with H2N2 or H3N2. The findings align with prior work (doi:10.3201/eid3001.230756; doi:10.1101/2025.03.30.25323419) and provide a valuable perspective by linking childhood immune imprinting to cross-reactive NA responses. However, the robustness of these conclusions depends heavily on the sample size per age or birth cohort. Without information on the number of sera tested in each group, it is difficult to determine whether the observed differences are statistically powered and generalizable, or potentially influenced by small sample bias. Including powered sample numbers would allow for more rigorous assessment of variability within groups and strengthen confidence in the reported correlations.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

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In this revision, the authors addressed the comments raised in the review, the manuscript is much improved. Specifically, the authors added the missing information on the study cohorts (acknowledging limitations of the small sample sizes) and included N2 NAI antibody data from the same study cohort, an essential analysis to demonstrate the specificity of the N1 imprinting. In addition, the authors also included new N5 NAI data, strengthening the manuscript.
Minor edits needed:
-Line 52, case numbers from the outbreak may continue to evolve, add a cutoff date for the case number cited in this report, e.g. “As of March 24, 2026, this outbreak resulted in xx infections in humans…..”
-Line 191: The threshold of protection and seropositivity level for NAI antibody titers are not well defined. Delete the word “seropositive”, just state “with only 21% of samples possessing NAI titer of 40 or higher”.
-Line 210: Can update the reference of the preprint to the same published paper:
Li, et al. Pre-existing cross-reactive immunity to highly pathogenic avian influenza 2.3.4.4b A(H5N1) virus in the United States. Nat Commun 16, 10954 (2025). https://doi.org/10.1038/s41467-025-66431-2
-Extended data figures 1 and 2: the Figure quality needs to be improved, currently the figure labels are not legible.

Reviewer #2

(Remarks to the Author)
The authors were responsive to reviewer's comments. I have no further suggestions.

REVIEWER COMMENTS

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Comments for consideration:
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2). Serum samples collected in 2005, numbers unknown with no details
3). Seasonal influenza infection sera.

Suggest the authors include a table to describe the critical parameters of the sera used, including the sample sizes in each of the birth cohorts studied and acknowledge the small sample size as a study limitation.

Response: We agree that a table further detailing the cohorts from which we obtained serum is useful; this has been added as **Extended Data Table 5**. We have additionally added a histogram as **Fig. 1a** showing the year of birth distribution for the 2017 cohort from which we conclude that baseline NAI titers are correlated with imprinting probabilities.

Additionally, we added a sentence at **lines 235–238** acknowledging that the sample sizes tested may be a limitation of our study. However, we believe that the findings from our 2017 cohort are generalizable for multiple reasons. First, we previously used this cohort to test for HA imprinting and have published these results; this cohort size was sufficient to statistically detect effects of HA group 1-level imprinting on cross-reactive H5 antibody responses (Garretson et al., Nature Medicine, 2025). Additionally, as shown in the histogram, we have serum collected from individuals across a broad year of birth range, with relative uniformity over time. These samples include 45, 33, and 77

individuals born during each of the IAV NA (co-)circulation birth cohorts (past–1956 [N1], 1957–1976 [N2], and 1977–present [N1+N2], respectively). Finally, our bootstrapping analysis was used to assess if the correlation with imprinting was robust and generalizable across our cohort, or if it may be driven by a subset of individuals. Across 1,000 bootstrap replicates—in which the data are randomly resampled with replacement, resulting in some data points being duplicated and others omitted—we still find strong correlations between N1 titers and N1 imprinting probabilities, which outperform other tested predictors.

2. The authors can reference a recent study reported by ZN Li et al (Population Immunity to Hemagglutinin Head, Stalk and Neuraminidase of Highly Pathogenic Avian Influenza 2.3.4.4b A(H5N1) viruses in the United States and the Impact of Seasonal Influenza on A(H5N1) Immunity | medRxiv). This study analyzed a larger sample size and investigated broader age groups and reported the prevalence of NA antibodies of A(H1N1)pdm09 and 2.3.4.4b H5N1 viruses with similar age-related patterns. A(H1N1)pdm09 virus infection in adults elicited significant rise of cross-reactive NAI antibody response to H5N1. In addition, they observed a significant rise of cross-reactive NAI antibody response to the NA of H5N1 in different age cohorts following recent vaccinations. This study also explored the similarity between the N1 NA epitopes of H5N1 and H1N1 virus which can contribute to the N1-imprinting effect.

Response: We thank the reviewer for this relevant piece of literature. We added **lines 208–210** to the Discussion section to address these findings in the context of our manuscript.

3. For the same set of serum samples used for Figure 1, suggest the authors test N2 NAI antibodies as a specificity analysis for the source of NA imprinting. It would also be interesting to see if the prevalence of N2 antibodies is correlated with N2-imprinting, and whether the two NA imprinting pathways can interact and affect each other. This may provide insights into the peak titers for people born during the 1990s (Figure 1) which as the authors discussed, cannot be explained by imprinting probabilities alone.

Response: We agree that it is important to confirm specificity of our N1 findings and that performing assays against an N2 NA would help to clarify this. We have now performed NAI assays against H6N2 viruses expressing the NAs of A/Hong Kong/1/1968 and A/Perth/16/2009 (H3N2 viruses) using sera from this cohort. These data have been added as **Extended Data Fig. 2**, showing limited correlations between N1 and N2 titers and unique birth year trends. This demonstrates that our NAI assay can detect antibodies against specific NAs. These data are discussed on **lines 153–164**. These data are in line with previous findings from Sook-San Wong's group, who found that individuals ≥65 years of age had the highest NAI titers against a 1968 H3N2 virus, while younger individuals had peak titers against viruses circulating around their time of birth (Liang et al., Nat Commun, 2024).

In our revision, we also performed assays to measure antibodies against the NA of an H5N5 virus (A6 genotype) that has circulated in birds in North America and recently

caused a fatal human infection in the United States (**lines 182–203; new Figure 4**). Consistent with the observation that viruses with N5 have not widely circulated in humans, we found low rates of seropositivity against the N5 NA, with markedly lower titers compared to N1 NAs. This suggests that the human population could potentially be more susceptible to H5 viruses that reassort to include N5 rather than N1.

4. In line 94-110, should describe how “N1 imprinting probabilities” is defined and how it differs from the “birth year” in the analysis, assuming that the N1 imprinting probabilities are also determined based upon the birth years.

Response: We thank the reviewer for this suggestion and agree that this would add clarity. We have now added an explanation of how imprinting probabilities are defined and calculated to this paragraph on **lines 114–118**.

Reviewer #2 (Remarks to the Author):

In this Brief Communication, Ort et al. demonstrate that individuals primed in childhood with H1N1 influenza viruses exhibit higher levels of antibodies cross-reactive with clade 2.3.4.4b H5N1 neuraminidase than those primed with H2N2 or H3N2. The findings align with prior work (doi:10.3201/eid3001.230756; doi:10.1101/2025.03.30.25323419) and provide a valuable perspective by linking childhood immune imprinting to cross-reactive NA responses. However, the robustness of these conclusions depends heavily on the sample size per age or birth cohort. Without information on the number of sera tested in each group, it is difficult to determine whether the observed differences are statistically powered and generalizable, or potentially influenced by small sample bias. Including powered sample numbers would allow for more rigorous assessment of variability within groups and strengthen confidence in the reported correlations.

Response: We thank the reviewer for their kind comments and suggestions. As discussed in our response to Reviewer #1’s comment #1, we have now added a table outlining the cohorts used in our study **Extended Data Table 5** as well as a histogram to show the year of birth distribution for our 2017 cohort **Fig. 1a**. This cohort include 33 individuals born during exclusive N2 circulation (1957–1976), the shortest of the IAV NA (co-)circulation birth cohorts, as well as 45 and 77 individuals from the other birth cohorts.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Response: We thank the ECR for their time spent reviewing our manuscript and Nature Communications for providing this valuable initiative!

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

In this revision, the authors addressed the comments raised in the review, the manuscript is much improved. Specifically, the authors added the missing information on the study cohorts (acknowledging limitations of the small sample sizes) and included N2 NAI antibody data from the same study cohort, an essential analysis to demonstrate the specificity of the N1 imprinting. In addition, the authors also included new N5 NAI data, strengthening the manuscript.

Minor edits needed:
-Line 52, case numbers from the outbreak may continue to evolve, add a cutoff date for the case number cited in this report, e.g. “As of March 24, 2026, this outbreak resulted in xx infections in humans…..”

The manuscript has been updated at **line 51** to add a cutoff date.

-Line 191: The threshold of protection and seropositivity level for NAI antibody titers are not well defined. Delete the word “seropositive”, just state “with only 21% of samples possessing NAI titer of 40 or higher”.

We have updated **line 191** to remove the word “seropositive.”

-Line 210: Can update the reference of the preprint to the same published paper:
Li, et al. Pre-existing cross-reactive immunity to highly pathogenic avian influenza 2.3.4.4b A(H5N1) virus in the United States. Nat Commun 16, 10954 (2025). https://doi.org/10.1038/s41467-025-66431-2

We have updated the reference on **line 210** to the published version of this paper.

-Extended data figures 1 and 2: the Figure quality needs to be improved, currently the figure labels are not legible.

We thank the reviewer for this suggestion; we have updated the **Supplementary Figures 1** and **2** to improve legibility, and additionally increased font size on some main figure texts which were previously difficult to read.

Reviewer #2 (Remarks to the Author):

The authors were responsive to reviewer's comments. I have no further suggestions.

We thank the reviewer for their prior feedback and for reviewing the revised manuscript.